INFLUENCE OF ATMOSPHERIC PRESSURE PLASMA TREATMENT ON EPITHELIAL REGENERATION PROCESS

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In the last decade, advances in atmospheric pressure plasma technology allowed development of new many applications. Non-thermal plasma systems have been used for surface sterilization and functionalization, blood coagulation and more recently for tissular regeneration. We report here the use of non-thermal plasma to create a specific atmosphere for epidermal regeneration. We have assessed local and systemic changes during epidermal regeneration under different therapeutic conditions, using a helium atmospheric pressure plasma jet.

Key words: Plasma medicine, reepitelization and tissue regeneration.

1. INTRODUCTION

Progresses in interdisciplinary sciences like biophysics, bioengineering and medical physics have brought many benefits in medical world, both for fundamental research and for clinical activities. Knowledge from physics and chemistry are now applied in biology, pharmacology, tissue engineering, surgery, biomaterial science etc. [1, 2].

Plasma technologies represent a good illustration of excellence in basic science transferred to medicine [3]. Few years ago non-thermal plasmas were mostly focused on surface modification by treatment or deposition to control materials biocompatibility or hemocompatibility. New applications have recently arisen, like tissue engineering or sterilization. The applications list of non-thermal plasmas is continued nowadays even further to direct use in medicine like treatment of living tissues, including wound healing and sterilization, blood coagulation or cell metabolism modification, with cancer treatment as target [4-9].


Plasma treatment has been checked out for its influence on biological molecules involved in cell adhesion, proliferation and differentiation [10, 11].

Regarding plasma effects on living cells it was found very different behaviour of living cells under direct cold plasma treatment, depending on cell type and plasma source power and composition. For instance, in malignant cell lines plasma was found to promote apoptosis and even necrosis, while applied on normal cell lines plasma stimulates cell adhesion and tissue regeneration [12, 13].

Nevertheless many features of non-thermal plasma use in medicine remain to be studied. We include here plasma physics aspects, biological effects, safety hazards or standardization of plasma sources operational parameters.

The purpose of present study is to compare spontaneous reepithelization characteristics versus plasma-assisted reepithelization. We have enhanced the reepithelization process by direct atmospheric pressure cold-plasma jet treatment. We have followed systemic parameters change: hematologic and biochemical parameters, and local features: oxidative stress markers and histology of skin in the above mentioned conditions.

2. MATERIALS AND METHODS

We have performed our experiments on Wistar rats and our protocol has been approved by the Ethics Committee of „Grigore T. Popa” University of Iasi. We have considered 3 groups, each one consisting of 10 healthy males, with body weight ranging from 320 to 380 grams. The groups have been considered as follows:

- \(M\) = control group, 12 rats without skin wounds;
- \(ANT\) = 12 rats with burn wounds which healed spontaneously (untreated group);
- \(APPJ\) = 12 rats treated with atmospheric pressure plasma jet directly on their skin wounds.

Before making the burn wounds and before the animals being sacrificed we have anesthetized them injecting intraperitoneal mixture of Ketamine (50 mg/kg) and Xilazine (10 mg/kg).

In order to assess reepithelization of the injured skin, we have imagined and designed a burn wound model on Wistar rat skin. Our aim was to create standardized, easy reproducible and quantifiable skin lesions involving entire epidermis and superficial dermis.

We performed chemical burns on the rat’s back skin (dorsal chest and lumbar region), four lesions each side of median line. The skin burn wounds were disc shaped, with clear borders, achieved by instilling of an acidic solution in a special plastic device, five mm in diameter of the contact aperture, so that the lesions had a
predetermined area (approximate 19.6 mm²). We have used sulphuric acidic solution because of its limited action along with the time, unlike alkaline solutions, whose effect in time might be unpredictable and more difficult to assess. We found the optimal acidic solution concentration and exposure time to achieve second degree, partial thickness skin burns, which means all epidermis and superficial dermis are damaged. Immediately after the skin lesions were done the skin looked normal in colour and texture, yet after five to ten minutes the burnt skin become brawn or dark red.

We have treated the burn skin wounds by directly irradiating them with an atmospheric pressure cold plasma jet (APPJ). The APPJ was generated by a dielectric barrier discharge in helium, at atmospheric pressure. The gas flows through a quartz cylinder having applied on the external wall the power electrode.

![Fig. 1 – Typical plasma discharge current and optical emission spectrum.](image)

The floating (passive) electrode was the living animal skin. The high voltage pulses have 4 kV amplitude and 2 kHz frequency (Fig. 1). Typical values of the peak current achieved are in the range of milliamperes. Maximal power density applied was 0.8 Watt/cm². Optical emission spectroscopy analysis showed that beside helium, our atmospheric plasma contained neutral and ionized molecular nitrogen, and active species such as atomic oxygen, singlet oxygen or hydroxyl radical (Fig. 1). The source of these species is the air that surrounds the plasma jet and further experiments are necessary in order to control this gas environment. The active species might interact with substrate molecules (keratinocyte surface proteins) changing their electrical charge and function. No excited nitrogen oxide (NO), incriminated for the proregenerative properties of plasma, was observed our APPJ.

At day 3, 8, 14 and 21 we have made the sacrifices in order to harvest blood and skin samples for biochemical and histological analysis respectively.
The integral central blood has been checked out for the following parameters: complete blood count (including leucocyte distribution); sugar, transaminases, urea, creatinine, uric acid; fibrinogen and C3 complement; oxidative stress markers: malondialdehyde, reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase.

We have used skin samples for:

– oxidative stress markers in skin homogenate (epidermis and dermis have been homogenised at 1500 rpm, for 5-10 minutes, in a Potter-Elvehjem homogenizer);

– for histological examination slices have been stained using different techniques: Hematoxylin-Eosin and Szekely.

The total follow up period was 21 days. Statistic analysis was performed with t-Student and Chi-square tests; we have used SPSS 13.0 and Microsoft Excel 1997.

3. RESULTS AND DISCUSSION

Hematologic parameters change:

In the untreated group (ANT, with spontaneously healed lesions) we note significant increase in total white blood cells, especially neutrophils, for all the follow up period, compared with control group (M, without burn lesions). Lymphocytes and monocytes slowly increased in the last 3 days (Table 1).

In the group with skin lesions treated with cold helium plasma (APPJ group) the WBC population slowly increased in the first three days, and decreased back to normal thereafter (Fig. 2), with polymorphonuclear cells dominating the leukocytic distribution in the first 3 days. In the following 18 days monocytes and lymphocytes clearly dominated the leukocytic distribution. The overall analysis showed statistically significant decrease in the cellular immune response (p<0.001) for the plasma treated group (APPJ) compared with untreated groups (ANT). This data suggest that directly treatment of injured skin with helium plasma might calm down the immune response via bactericidal effect or it may directly suppress the immune cells activation by modulating complex mechanisms.

Fig. 2 – WBC count, C3 complement and fibrinogen dynamic, for biochemical control group (M), untreated lesions (ANT) and for directly treated lesions with helium plasma (APPJ).
Table 1
Leucogram at day 21

<table>
<thead>
<tr>
<th></th>
<th>M group</th>
<th>ANT group</th>
<th>APPJ group</th>
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<tbody>
<tr>
<td><strong>Granulocytes</strong></td>
<td>M(%)±DS</td>
<td>24.02±0.52</td>
<td>21.4±0.94</td>
</tr>
<tr>
<td></td>
<td>M(x10^9/L)</td>
<td>(1.04x10^9/L)</td>
<td>(2.74x10^9/L)</td>
</tr>
<tr>
<td><strong>Monocytes</strong></td>
<td>M(%)±DS</td>
<td>8.45±0.76</td>
<td>9.03±0.11</td>
</tr>
<tr>
<td></td>
<td>M(x10^9/L)</td>
<td>(0.43x10^9/L)</td>
<td>(1.17x10^9/L)</td>
</tr>
<tr>
<td><strong>Lymphocytes</strong></td>
<td>M(%)±DS</td>
<td>62.75±0.92</td>
<td>69.0±1.11</td>
</tr>
<tr>
<td></td>
<td>M(x10^9/L)</td>
<td>(2.35x10^9/L)</td>
<td>(9.25x10^9/L)</td>
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Biochemical analysis:
Non-specific inflammation markers (fibrinogen and C3 component of serum complement) significantly increased all along the follow up period (p<0.001) in the untreated group (ANT) compared with control group (M). In the plasma treated group (APPJ), C3 complement and fibrinogen slowly increased in the first 3 days and decreased to normal after 8 days of direct plasma treatment (Fig. 3). The blood sugar, transaminases, urea, creatinine and uric acid did not significantly differ between nontreated and plasma treated groups.

In order to assess local and systemic oxidative stress we have checked out some specific markers both in skin homogenate samples and in serum. The oxidative stress markers are: malondialdehyde and reduced glutathione level; glutathione peroxidase, superoxide dismutase and catalase activity [14-18].

In ANT and APPJ groups we noted mild increase in systemic oxidative stress markers, compared with M group. The highest level was at day 3 after which they slowly decreased to normal till the end of our experiment.

In the skin homogenates of untreated burns group (ANT) we found important oxidative stress (p < 0.001) at day 3, compared with control group, which then slowly decreased to normal at day 8, 14 and 21.

In the plasma treated group (ATJP), local oxidative stress was very important (p < 0.001) all along the experiment: oxidative markers in skin homogenates formed a plateau of abnormaly high values for all the follow up period.

According to spectrometric analysis, our plasma contained high levels of hydroxyl radical, singlet oxygen and ionized nitrogen, which might trigger and intensify local oxidative chain reaction. High level oxidative stress kills bacteria and stimulates reepithelization. There was ipothesized that high oxidative stress may stimulate some matrix metaloproteinases expression hence stimulating keratinokite migration.

Histology:
At day 3, 8, 14 and 21, specimens were taken from the injured skin for the histologic analysis. We have used panoptic Hematoxilin-Eosin and Szekely stain for our specimens.
At day 3:
- in the self healed lesions we noted epidermal necrosis, superficial and deep dermal connective tissue hyalinization (through collagenic degeneration); deeply, in the surrounding dermis and beneath the burnt epidermis, there was faint perivascular inflammatory infiltrate;
- in the helium plasma treated lesions we noted the same aspect, with epidermal necrosis and dermal hyalinization but the perivascular infiltrate in the deep dermis was more important.

At day 8:
- the self healed lesions presented with a stack of fibrin and leucocytes in the superficial, denuded dermis; the epidermis begun to regenerate from the lesion margins;
- the lesions treated with helium plasma presented with a thinner stack of fibrin and inflammatory cells covering the wound crater, the inflammatory infiltrate in the superficial and deep dermis was less important compared with that in self healed lesions; the epidermis begun to regenerate energically from the wound margins, it was thicker compared with that in self healed lesions.

At day 14:
- in the self healed lesions the epidermis was thin and the superficial dermis more homogenized, with collagen fibers horizontalized;
- in the lesions treated with helium plasma, the epidermis was heterogeneously thicker and corrugated and the dermis condensed. The epidermis thickness was significantly more important than in the self healed lesions.

At day 21 (Fig. 3):
- untreated lesions presented with a thin, linear epidermis and superficial dermis with wavy collagen fibers (restored) similar to the adjacent normal region;
- plasma treated lesions presented with corrugated and thick epidermis, with a sinuous basement membrane and well represented spinous and corneum layers; the dermis beneath still remained hyalinated with collagen fibers condensed and poor monocytic infiltrate.

Fig. 3 – Histological aspect at day 21: self healed (a) and plasma treated lesions (b) (HE stain, magnification x 100).
5. CONCLUSIONS

We have achieved accelerated reepithelization in the plasma treated skin lesions. The overall analysis of hematologic, biochemical and histological parameters showed positive correlation. At day 21, although hematology and biochemistry showed normal values compared to control, the histological images showed abnormal epidermis and subjacent dermis regeneration under direct plasma irradiation. It was interesting that under direct plasma irradiation the inflammation parameters still remained at low levels while local oxidative stress, keratinocyte and fibrocyte response seemed to be quiet accelerated.

Reepithelization is just a part of the skin regeneration process, which recruits cellular and humoral components, with the aid of epidermal and dermal interaction via signal molecules.

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